APPLICATIONS OF TIME-RESOLVED FLUOROIMMUNOASSAY TO DETECT MAGNETIC BEAD CAPTURED *ESCHERICHIA COLI* O157:H7

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ABSTRACT

A time-resolved fluorescence technique was developed to detect Escherichia coli *O157:H7 in ground beef burger. After a 4.5 h enrichment period, streptavidin coated magnetic beads conjugated with biotin-labeled anti E. coli O157:H7 were used to capture the bacteria. The bacteria were, at the same time, also labeled by a nonfluorescent, europium (Eu)-tagged anti-E. coli O157:H7 antibody. The sandwiched bacterial complexes were then concentrated using a magnetic particle concentrator and washed to remove other solution components. Upon addition of an enhancement buffer, the Eu-labels were then released from the antibodies and chelated to nitrilo-triacetic acid (NTA) and trioctylphosphine oxide (TOPO) to form highly fluorescent Eu-$(2$-$NTA)_3(TOPO)_{2-3}$ micellar complexes. Delayed fluorescence associated with these complexes was measured and its intensity was used to estimate the original bacterial concentration spiked in hamburger. This approach was applied to detect E. coli O157:H7 spiked in hamburgers. The results indicated this method is able to detect ~ 1 CFU/g of the bacteria after a brief enrichment for four and half hours at 37C. Specificity studies indicated that the approach exhibited no or limited cross reactivity to* Salmonella typhimurium, E. coli *K-12 or* Shigella dysenteriae

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spiked in hamburgers. Thus, the developed approach may be used as a rapid screening procedure for E. coli *O157 bacteria in foods.*

INTRODUCTION

Rapid detection of specific pathogenic bacteria is essential to assure the food safety required by consumers. Many standard microbiological culture methods have the necessary specificity and sensitivity for pathogenic bacteria detection. However, the requirements of long culture times, specialized facilities and confirmation by biochemical tests limit their general applications for practical purposes.

Efforts to develop alternative methods include but are not limited to ATP bioluminescence (Sharpe *et al.* 1970), antibody directed fluorescent microscopy (Tortorello and Stewart 1994), polymerase chain reaction (Bej *et al.* 1994), quartz crystal microbalance biosensor (Minunni *et al.* 1995), electrochemical enzyme immunoassays (Brewster *et al.* 1996), etc. However, these methods do not have the necessary sensitivity to directly detect the infectious dosages of *E. coli* O157:H7, e.g., < 1 CFU/g in hamburger. Thus, culture enrichment is needed to increase pathogen concentration and enhance the probability of pathogen detection. One attractive alternative (Molday *et al.* 1977) to rapidly and effectively separate and concentrate targeted pathogens in complex food matrices is the use of immunomagnetic beads (IMB). IMBs have been applied in several rapid methods for the capture of bacteria prior to analysis (Fratamico *et al.* 1992; Olsvik *et al.* 1994). Several recent studies have shown the advantage of concentrating targeted bacteria by IMB during signal detection steps (Gehring *et al.* 1996; Yu and Bruno 1996). In the past few years, we have incorporated IMB separation and concentration steps in an effort to develop new biosensor-based detection processes for *Escherichia coli* O157:H7 including digital fluorescence microscopy for intact cells (Tu *et al.* 1998), ATP biolumi-nescence for viable cells (Tu *et al.* 1999) and light addressable potentiometric sensor (Tu *et al.* 2000).

The involvement of $4f$ orbitals in the electronic structure of lanthanide cations (La) such as europium permits a transfer of excitation energy from ligands to central La cations prior to the emission of ion fluorescence that is characteristic by a relative long fluorescence half-life (~ 50 to 1000 μsec) and a considerable Stoke's shift (>200 nm) between the absorption and emission maxima. In contrast, the fluorescence half-life and Stoke's shift of common organic and biochemical compounds are in the range of 1 to 1000 msec and 20 to 100 nm, respectively. Thus, with a pulsed excitation, the fluorescence of La may be easily filtered out from the interference fluorescence and scattered excitation light by delaying the emission measurement. In addition, the quantum

yield of La-chelates is usually quite high, e.g., 0.18 for Eu-(4,4,4-trifluoro-[2-thienyl-1,3-butanedionato]) at 614 nm (Halverson *et al.* 1964).

O'Haver and Weinfordner (1966) applied a pulsed flash lamp as an excitation light source and an electronic gate to switch on the photomultiplier only during the dark period, to detect the presence of 0.1 ng/mL of many drugs and metabolites using time-resolved fluorescence (TRF) measurement. A combination of time delayed fluorescence and the unique properties of La-chelates has led to the development of a new technique called Dissociation-Enhanced Lanthanide Fluoroimmunoassay (DELFIA). In this technique, antibodies are modified to contain binding groups capable of forming very low fluorescence La-complex. The modified antibodies are used to capture target species. The antibody-bound La cations are then extracted out by an "enhancement solution" that contains chelates capable of forming strongly fluorescence products.

In our current study, we investigated the possibility of applying an IMB process to time-resolved fluorescence (TRF) of lanthanide-series (La) cations for the detection of *E. coli* O157:H7. The long half-life of lanthanide cations allows the fluorescence of La cations to be measured after the decay of background emissions (Soini and Lövgren 1987). The quantum yield of complexed-La cations exhibits a strong dependence on the nature of the ligands (Sinha 1971). This dependence allows the development of the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) in which La cations in low fluorescent antibody-complexes were extracted by free ligands to form strongly fluorescent La-chelates (Lövgern *et al.* 1985).

In practice, targeted *E. coli* O157:H7 cells formed sandwiched complexes with IMB and Eu-labeled antibodies. The complexes were then separated and concentrated by the use of a magnetic particle concentrator (MPC, Dynal A.S., Norway). Upon addition of an enhancement solution, the intense fluorescence of Eu-chelates was measured. This approach allowed us to detect low levels of *E. coli* O157:H7 in hamburger ($\sim$ 1 CFU/g) after a 4.5 h enrichment at 37C.

MATERIALS AND METHODS

Culture of Bacteria

E. coli O157:H7 (strain 1409), *E. coli* O157:NM (strain MF 13180), *E. coli* K-12, *Shigella dysenteriae* (strain ATCC 29026) and *Salmonella typhimurium* DT104 (strain M26-202) were grown separately in brain-heart infusion (BHI) broth at 37C with 160 rpm shaking to yield cell concentration of approximately 10^9 CFU/mL. After 18 h, the cultures were placed on ice to halt growth and were diluted in PBS for enumeration on triplicate Sorbital-MacConkey plates.

Growth of Bacteria in Hamburger

Freshly prepared bacterial cultures were serially diluted in EC media (DIFCO) containing 4 μg/mL of sodium novobiocin (Sigma, St. Louis, MO). Eighty-five percent lean hamburger was purchased from a local supermarket, formed into 25 g patties and placed into Whirl-pak stomacher bags with filters (Nasco, Fort Atkinson, WI). Twenty-five mL bacterial suspensions were added and manually mixed into the hamburger patties to achieve inoculum levels of 0, 1, 10 and 100 CFU/g. The hamburger suspensions were then incubated at 37C with 160 rpm shaking. At the end of a 4.5 h incubation, the hamburger was homogenized in a Seward Stomacher 400 (Seward Co. England) for 2 min at normal speed setting. A volume of 2 mL of filtrate per bag was removed and filtered for a second time using SMA columns (Fisher Scientific) to remove small particulate matter.

Capture of Bacteria

E. coli O157:H7 cells were allowed to form sandwiched complexes with biotinylated anti-*E. coli* O157:H7 antibodies conjugated with streptavidin coated magnetic beads and with Eu-labeled anti-*E. coli* O157:H7 antibodies. For magnetic bead conjugations, streptavidin coated Dynabeads (Dynal A.S., Norway) were concentrated/washed twice with PBS + 0.1% BSA and allowed to interact with goat polyclonal biotinylated anti-*E. coli* O157:H7 (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at a concentration level of 10 μg per mg of beads for 30 min at room temperature with gentle rocking. The antibody conjugated magnetic beads (6 $\times$ 10^8 beads per mg) were washed four more times and stored for up to one month at 4C in PBS containing 0.1% BSA. Prior to use for bacterial capture, the antibody coated beads were concentrated with a MPC and washed once and resuspended in TRF Assay Buffer (PerkinElmer Wallac, Turku, Finland), Tris-HCl buffered NaCl solution containing NaN$_3$, bovine serum albumin (BSA), bovine gamma globulins, Tween 40 and diethylenetriaminepenta-acetic acid (DTPA) supplemented with different concentrations of Tween 20 (Sigma, St. Louis, MO).

TRF Measurement

Thirty μL of antibody-coated beads (1.8 $\times$ 10^7 beads), 100 μL of Eu-labeled anti-*E. coli* O157 antibodies (KPL, Gaithersburg, MD; labeled by PerkinElmer Wallac, Norton, OH) diluted to 100 ng per mL in Assay Buffer plus 0.1% Tween 20 and filtered with a Nalgene 0.45 μm syringe filter, 100 μL of bacterial suspensions and 770 μL of Assay Buffer containing 0.1% Tween 20 were combined in a 1.5 mL microcentrifuge tube. The mixture was incubated for 1 h at room temperature with gentle rocking. The sandwiched bacterial

complexes were then concentrated and washed utilizing a MPC and Washing Concentrate (diluted 1:25 in distilled water; PerkinElmer Wallac, Turku, Finland) containing a Tris-HCl salt solution buffered to pH 7.8, Tween 20 and a preservative. After the sixth wash, the complexes were suspended in 100 μL enhancement solution (PerkinElmer Wallac, Turku, Finland) containing Triton X-100, acetic acid and chelates, added to the wells of a black polystyrene 96-well microplate (COSTAR 3915) and incubated for 5 min at room temperature on a plate shaker (Thermolyne, Dubuque, IA). The delayed fluorescence was measured utilizing a VICTOR^2 1420 Multilabel Counter (PerkinElmer Wallac, Turku, Finland) and the parameter settings for Eu.

Materials and Chemicals

Hamburger patties were purchased from local supermarkets. Eu-labeled anti-*E. coli* O157 antibodies was customer-synthesized by PerkinElmer. The extent of labeling was 11.9 Eu per protein molecule. TRF-related chemicals and reagent solutions were purchased from PerkinElmer. All other chemicals were of analytical grades.

RESULTS AND DISCUSSION

General Experimental Design

As described, the purpose of this study was to investigate the possibility of combining immunomagnetic separation/concentration and time-resolved fluorescence technology for the detection of *E. coli* O157:H7. Biotin-labeled anti-*E. coli* O157:H7 antibodies after conjugated to streptavidin coated magnetic beads, were employed as the bacterial capture mechanism. Europium-labeled anti-*E. coli* O157:H7 antibodies were used to report the presence of targeted bacteria. The combination of immunomagnetic beads and La-related TRF-DELFIA for bacterial detection is illustrated in Fig. 1.

Sensitivity of the Detection

The design described in Fig. 1 was applied to detect cultured *E. coli* O157:H7 using europium-labeled antibody. The signal to background ratio of a DELFIA may be affected by several factors, including the delay time setting, the detergent concentration, etc. As shown in Fig. 2, samples with 1000 CFU/mL of the bacteria obtained from broth cultures, exhibited a signal intensity of ~10,000 counts per second (CPS) and a ratio of signal to background greater than 2. Both the signal intensity and the ratio increased as the bacterial concentration increased. As expected, the total fluorescence

intensity decreased as the delay time increased (Table 1). The signal to background ratio increased and then reached a plateau as the delay time increased. A delay time between 400 to 500 msec was chosen to assure better than 2 of the ratio and yet with sufficient signal intensity for reliable determinations.
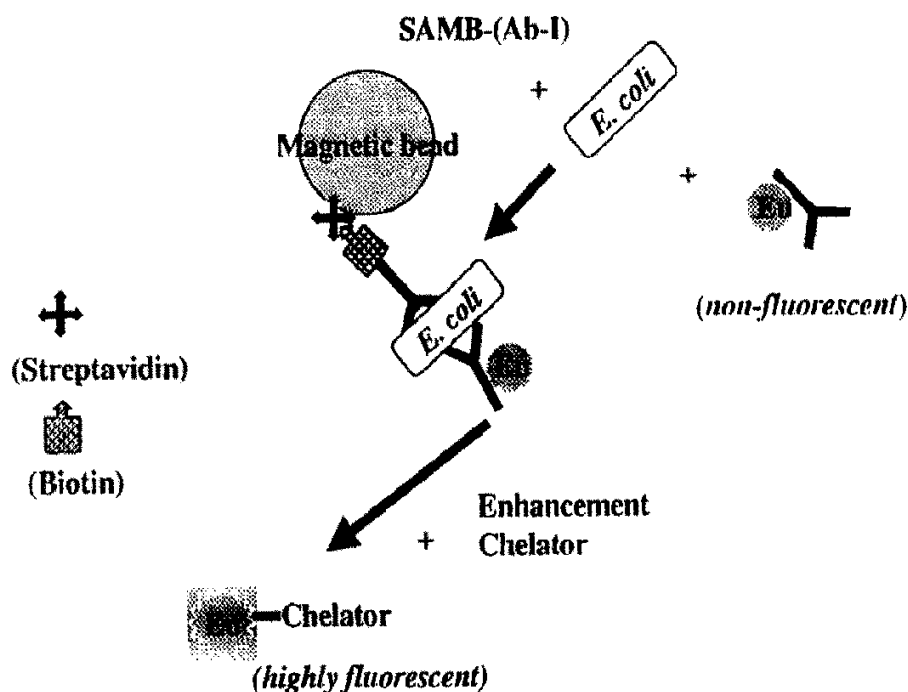

FIG. 1. DESIGN FOR THE CAPTURE AND DETECTION OF *E. COLI* O157:H7
The capture and concentration of the bacteria by immunomagnetic beads and Eu-labeled antibody against the same bacteria was described in text. After the removal of other contaminants by washing, an enhancement solution was added to release the Eu-labels from the antibodies. The enhanced fluorescence was then measured as described.

In addition to minimizing the nonspecific binding of Eu-complexes, Tween detergents in the assay and washing buffers could also minimize the exposure of the Eu complexes to aqueous environment. The hydrophobic core of the mixed micelles formed between the detergent and the complexes, would enhance the fluorescence intensity associated with the Eu ions. Although the commercial assay buffer contained unspecified concentration of Tween, the formulation was primarily optimized for the detection of small molecules. To determine whether additional detergent should be included in the buffer, we have supplemented the

PerkinElmer Wallac buffer with additional 0 to 0.1% (v/v) of Tween 20 as suggested by Batina *et al.* (1997). Indeed, the addition of Tween 20 to the assay buffer significantly increased the intensity and the ratio of signal to background over a wide range of the bacterial concentration (Table 2). The enhancement in intensity and the increase in the ratio appeared to peak off at ~0.1% of Tween 20. Thus, additional 0.1% of Tween 20 was routinely added to the commercial assay buffer.
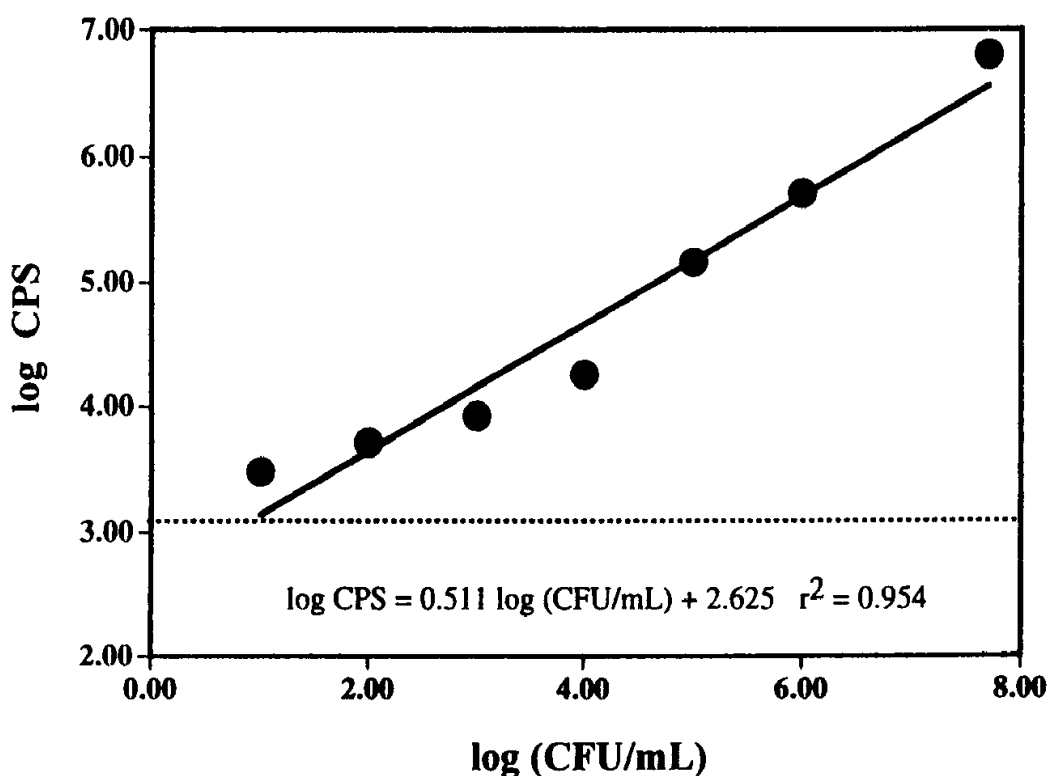

$$\log CPS = 0.511 \log (CFU/mL) + 2.625 \quad r^2 = 0.954$$

FIG. 2. SENSITIVITY OF THE TRF-DELFIA DETECTION

Stationary-phase cultures of *E. coli* O157:H7 obtained in BHI media were serially diluted to the indicated concentration levels shown on X-axis. The bacteria were then captured and processed for TRF-DELFIA measurements as described in text. The measured fluorescence intensities in unit of counts per second (CPS) were recorded. The dash line indicated the background obtained with the suspensions containing the beads and Eu-labeled antibodies but without bacteria after the same procedure. The data shown represent averages of 3 experiments with errors as ±5%. The equation and the square of correlation of fitted line is inserted in the figure.

TABLE 1.
DELAY TIME FOR FLUORESCENCE MEASUREMENT OF Eu CHELATES[1]

CFU/mL	100 msec	200 msec	400 msec	500 msec
		Intensity	(CPS)	
0	5985	5145	2148	1822
10^3	9647	9430	5325	5500
10^4	39180	34794	21756	22361
10^5	186916	166650	104882	104923

[1] Solutions containing *E. coli* O157:H7 with indicated CFU/mL were treated with immunomagnetic beads and Eu-labels as described for TRF-DELFIA measurements with different delay time settings. The data represent the averages of 3 experiments with less than 5% errors.

TABLE 2.
EFFECTS OF ADDITIONAL TWEEN 20 TO THE FLUORESCENCE MEASUREMENT[1]

E. coli O157:H7 (CFU/mL)	Assay Buffer[2]	Assay Buffer + 0.1% Tween 20
	Intensity	(S/B ratio)
0	7318	3172
10^3	8020 (1.1)	6880 (2.2)
10^4	13318 (1.8)	9750 (3.1)
10^5	17452 (2.4)	14561 (4.6)
10^6	58660 (8.0)	54818 (17.3)
10^7	528824 (72)	476590 (150)
10^8	1836129 (251)	1478355 (466)

[1] *E. Coli* O157:H7 grown overnight in BHI, diluted in assay buffer with or without additional 0.1% Tween 20. The cells were then treated as described for TRF-DELFIA measurements. S/B refers to the ratios of detected signal associated with *E. coli* containing samples to that associated with no bacteria.

[2] Assay buffer was obtained from PerkinElmer Wallac.

Detection of *E. coli* O157:H7 Spiked in Hamburger

The developed process was applied to detect *E. coli* O157:H7 in hamburger systems. Hamburger patties were spiked with different dosages (1, 10 and 100 CFU/g) of the bacteria and then enriched in EC medium containing novobiocin (20 μg/mL) for 3.5, 4.5, 5 and 6 h at 37C. After capture and concentration by the use of immunomagnetic beads and the Eu-labeled antibody, the dissociation enhanced fluorescence of Eu-complexes was measured. For the case of spiking with 1 CFU/g, 3.5 h enrichment failed to generate a ratio of signal to background higher than 2. After 4.5 h enrichment, the results shown in Fig. 3 were obtained. Clearly, the signal to background ratio increased to about 10 for hamburger spiked with 1 CFU/g of *E. coli* O157:H7 after a 4.5 h enrichment at 37C. According to the Pathogen Growth Model of Buchanan and Whiting
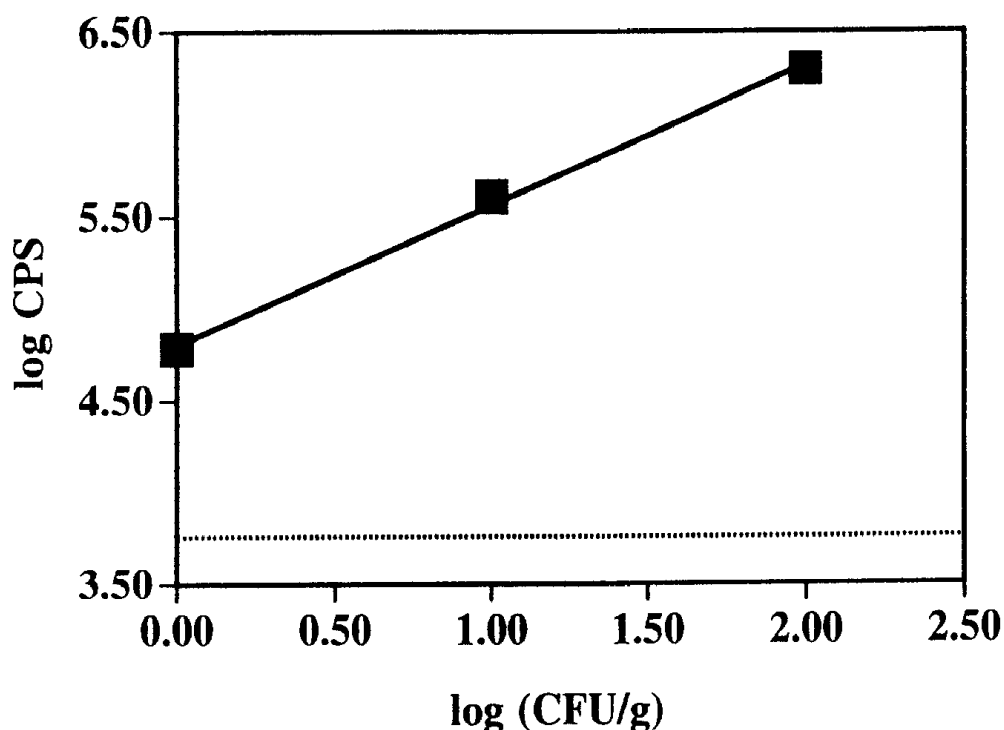

FIG. 3. SENSITIVITY OF THE TRF-DELFIA DETECTION OF *E. COLI* O157:H7 SPIKED IN HAMBURGER

Hamburger patties were spiked with indicated levels of *E. coli* O157:H7. The samples were then enriched in the novobiocin-containing EC media for 4.5 h as described in text. After IMB capture and concentration, the TRF-DELFIA signal intensities were measured. The data shown represent averages of 3 experiments with errors as $\pm$ 5%.

(http://www.arserrc.gov/mfs/pathogen.htm), this spiking level would yield ~10^4 CFU/mL of *E. coli* O157:H7. Since the detected fluorescence of *E. coli* O157:H7 in hamburger broth incubated for 4.5 h was comparable to that associated with 10^4 CFU/mL (Fig. 2), under the applied enrichment conditions, there was little, if any, inhibition of the growth of spiked *E. coli* O157:H7 from other bacteria already present in the hamburger.

Cross Reactivity of Developed Procedure

It has been reported that several bacteria belonging to the genus *Escherichia*, including *Escherichia hermanii*, *E. coli* O148:NM, and *E. coli* O117:H27 and group N *Salmonella* cross react with the polyclonal anti-*E. coli* O157 antibodies (Aleksic *et al.* 1992; Perry and Bundle 1990). This cross reactivity may increase the chance of reporting false positives for *E. coli* O157:H7. However, as pointed out by Goodridge *et al.* (1999), detection of these false-positive organisms may be advantageous if the organisms are pathogenic.

To gain some insight on the cross reactivity of the developed procedure, we have spiked hamburger patties with the same levels of different bacteria. After enrichment in the novobiocin-containing EC medium, the samples were treated with the immunomagnetic beads and Eu-labeled antibodies following the same experimental protocol as the immunoassays for *E. coli* O157:H7. The delayed fluorescence intensities associated with the bacteria were then determined. The developed procedure exhibited almost no cross reactivity toward *Shigella dysenteriae* (Fig. 4A), *Salmonella typhimurium* DT 104 (Fig. 4B) and *E. coli* K12 spiked in hamburger. However, considerable cross reactivity was found with spiked *E. coli* O157:NM. The results imply that antibodies used in the assay exhibit a specificity toward O157 bacteria and the novobiocin-containing EC media may prevent the growth of non-*E. coli* bacteria. To test the latter possibility, the developed procedure was applied to pure cultures of *Salmonella typhimurium* obtained from 18 h growth in BHI broth. This replacement of the growth media induced a minimal cross reactivity toward *Salmonella typhimurium* (Table 3). Thus, the antibody-specificity, the short enrichment time (4.5 h), together with the use of novobiocin that minimizes the growth of non-*E. coli* bacteria present in the hamburger constitute the basis for the effectiveness of screening *E. coli* O157:H7 by our developed procedure.
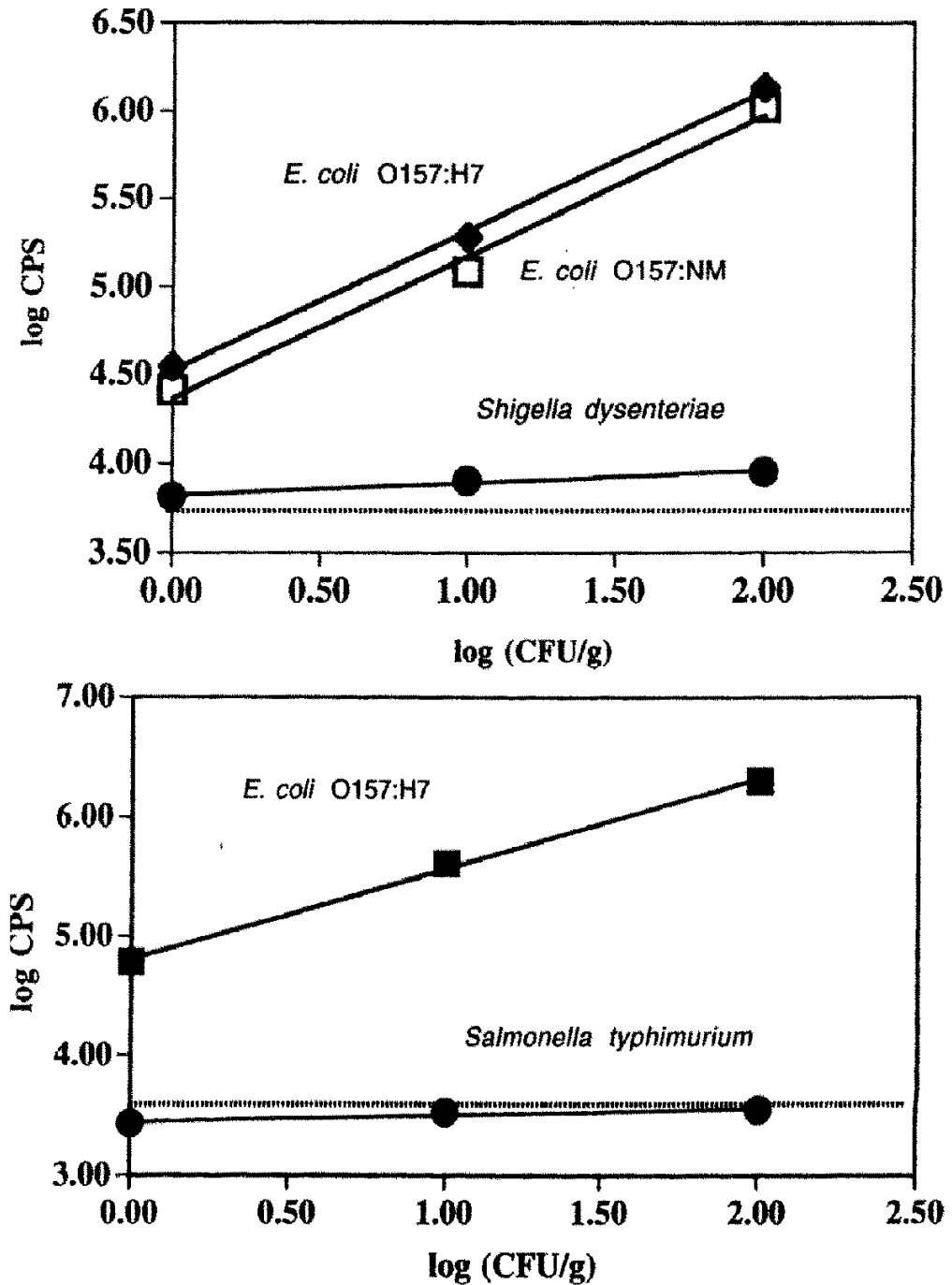
FIG. 4. CROSS-REACTIVITY OF DEVELOPED PROCEDURE TO OTHER BACTERIA
SPIKED IN HAMBURGER

Hamburger patties were spiked with indicated levels of *Shigella dysenteriae* (4A) and *Salmonella typhimurium* DT 104 (4B). The samples were then treated as described in Fig. 3. Not shown are the results obtained with *E. coli* K-12 which exhibited no cross reactivity to the procedure. The data shown represent averages of 3 experiments with errors as ± 5%.

TABLE 3.

CROSS REACTIVITY OF DEVELOPED PROCEDURE TO *SALMONELLA TYPHIMURIUM*[1]

CFU/mL	*Salmonella typhimurium* (CPS)	*E. coli* O157:H7 (CPS)
0	1132	1132
10^3	1546	5084
10^4	3053	43444
10^5	5853	417773

[1] *E. coli* O157:H7 and *Salmonella typhimurium* grown separately overnight in BHI, diluted in assay buffer plus 0.1% Tween 20 and fluorescence measured using developed TRF-DELFIA. The data shown represent averages of 3 experiments with errors as ±5%

CONCLUSIONS

The application of TRF-DELFIA to detect pathogenic *E. coli* O157:H7 (strain B1409) spiked in hamburger has been successfully demonstrated in our current study. The specificity of the developed procedure for *E. coli* O157:H7 detection in hamburger is enhanced by the use of novobiocin-containing EC broth prior to the capture of the bacteria by the antibodies. The sensitivity of the detection, < 1 CFU/g after a 4.5-h enrichment, is achieved by the use of Eu-chelates through a TRF-DELFIA approach that minimizes background fluorescence and enhances target quantum yield. The process has recently been modified in our lab to utilize a 96-well format and is, therefore, capable for high throughput assays of targeted pathogens. However, the practical value of developed procedure cannot be fully determined until more food matrices and bacterial stains are tested under different storage/stress conditions. Nevertheless, the developed procedure has the potential to become a rapid, sensitive and specific screening method for the presence of *E. coli* O157:H7.

The life-time and wavelength of lanthanide cations are quite different (Soini and Lövgren 1987). In principle, the developed procedure may be modified to simultaneously detect either different targeted bacteria or few parameters of a selected bacterium. For the former application, antibodies for different bacteria will be labeled with different La-cations. For the latter, the antibodies against selected parameters associated with the targeted bacteria can be labeled with different La-cations. With this approach, it is conceivable that detection of multiple pathogenic bacteria or further confirmation of the presence of a particular pathogen may be achieved. This possibility is currently being evaluated in our laboratories.

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